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Disaggregation of *Methanosarcina* spp. and Growth as Single Cells at Elevated Osmolarity

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The effect of medium osmolarity on the morphology and growth of *Methanosarcina barkeri*, *Methanosarcina thermophila*, *Methanosarcina mazei*, *Methanosarcina vacuolata*, and *Methanosarcina acetivorans* was examined. Each strain was adapted for growth in NaCl concentrations ranging from 0.05 to 1.0 M. *Methanosarcina* spp. isolated from both marine and nonmarine sources exhibited similar growth characteristics at all NaCl concentrations tested, demonstrating that these species are capable of adapting to a similar range of medium osmolarities. Concomitant with the adaptation in 0.4 to 1.0 M NaCl, all strains disaggregated and grew as single cells rather than in the characteristic multicellular aggregates. Aggregated cells had a methanochondroitin outer layer, while disaggregated single cells lacked the outer layer but retained the protein S-layer adjacent to the cell membrane. Synthesis of glucuronic acid, a major component of methanochondroitin, was reduced 20-fold in the single-cell form of *M. barkeri* when compared with synthesis in aggregated cells. Strains with the methanochondroitin outer cell layer exhibited enhanced stability at low (<0.2 M NaCl) osmolarity and grew at higher temperatures. Disaggregated cells could be converted back to aggregated cells by gradually readapting cultures to lower NaCl (<0.2 M) and Mg^{2+} (<0.005 M) concentrations. Disaggregated *Methanosarcina* spp. could also be colonized and replica plated with greater than 95% recovery rates on solidified agar basal medium that contained 0.4 to 0.6 M NaCl and either trimethylamine, methanol, or acetate as the substrate. The ability to disaggregate and grow *Methanosarcina* spp. as viable, detergent-sensitive, single cells on agar medium makes these species amenable to mutant selection and screening for genetic studies and enables cells to be gently lysed for the isolation of intact genetic material.

Methanogenic biodegradation of organic polymers occurs in a variety of anaerobic environments that include freshwater and marine sediments, ruminal and intestinal tracts of animals, and anaerobic waste digesters (2, 10). One group of methanogenic *Archaea*, the acetate-utilizing genus *Methanosarcina*, has a pivotal role in microbial consortia since up to 70% of the methane produced from polymer degradation in sediments and digesters is derived from the methyl moiety of acetate (9, 17). *Methanosarcina* spp. have been isolated from freshwater and marine environments, and they exhibit diverse morphologies. The *Methanosarcina* spp. isolated from marine environments, *Methanosarcina acetivorans* and *Methanosarcina frisia*, grow as osmotically fragile single cells that are surrounded by a protein S-layer (3, 21). The former species also forms communal cysts that yield large numbers of single cells when ruptured (21). In contrast, *Methanosarcina* spp. isolated from nonmarine environments grow as osmotically stable, multicellular aggregates embedded in a heteropolysaccharide matrix. This matrix is composed of methanochondroitin that is synthesized as an outer cell layer adjacent to the protein S-layer (15, 25). One exception is *Methanosarcina mazei*, which typically grows as aggregates but can disaggregate and grow as osmotically sensitive single cells or communal cysts similar to those described for *M. acetivorans* (19). *Methanosarcina barkeri* FR-19 is also reported to spontaneously disaggregate in substrate-depleted medium, but these cells exhibit low viability (4).

Most of the physiological, biochemical, and bioenergetic studies of the *Methanosarcina* spp. have been conducted on nonmarine species (10). However, genetic studies of these species have been impeded by the aggregating properties of the cells. Although *Methanosarcina* spp. have been grown previously as colonies on solidified medium, aggregation prevents colonization from single cells, which is required for effective mutant selection or screening (5, 7, 13). In addition, the harsh mechanical treatment required to rupture the resilient methanochondroitin outer layer formed by aggregated cells precludes the isolation of intact high-molecular-weight DNA and RNA for molecular genetic studies. Conditions that promote disaggregation of *M. mazei* to single cells have been reported previously (6, 16, 30). These cells are viable and can be maintained as single cells by the inclusion of divalent cations or high substrate concentrations in the medium. Harris (6) showed that the single-cell form of *M. mazei* can be grown with high efficiency on agar medium. However, the conditions reported are ineffective for growing disaggregated single cells of other aggregating *Methanosarcina* spp. A disaggregating enzyme from *M. mazei* culture supernatant, which has been characterized as an endopolysaccharide hydrolase, can disaggregate some strains of *M. barkeri* and *Methanosarcina thermophila* (6, 16, 31). Although this approach has been successfully used to extract DNA from several strains of *M. barkeri*, the disaggregated cells are not viable for plating (6).

In a previous study, we reported that *M. thermophila*, a nonmarine aggregating species, will adapt and grow in marine medium (0.4 M NaCl) by accumulating intracellular compatible solutes (26). Concomitant with osmotic adapta-

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tion, *M. thermophila* disaggregates and grows as single cells (23). Herein, we examine the ability of four additional *Methanosarcina* spp. to adapt and grow in media with NaCl concentrations ranging up to 1.0 M. The concomitant transition from methanochondroitin-forming aggregates to viable single cells that lack the heteropolysaccharide outer cell layer is shown to be a global phenomenon exhibited by all *Methanosarcina* spp. tested. Conditions that promote disaggregation and reaggregation of *Methanosarcina* spp. are described. We also report conditions for the maintenance of *Methanosarcina* spp. as viable, detergent-sensitive single cells for the isolation of intact genetic material and high-efficiency colonization on solidified medium.

MATERIALS AND METHODS

Bacterial strains. Sources for *M. thermophila* TM-1 (= DSM 1825), *M. mazei* S-6 (= DSM 2053), *M. barkeri* MS (= DSM 800) and 227 (= DSM 1538), and *M. acetivorans* C2A (= DSM 2834) and C2E have been described previously (24, 25). *M. barkeri* UBS (= DSM 1311), *M. mazei* LYC and *M. barkeri* W were provided by R. A. Mah. *M. barkeri* Fusaro (= DSM 804) and *Methanosarcina vacuolata* Z-761 were provided by G. Gottschalk.

Liquid media and culture maintenance. Sterile media were prepared anaerobically in an atmosphere that contained N_2-CO_2 (4:1) by a modification of the Hungate technique (2). All gasses were passed through a column of reduced copper turnings at 350°C to remove traces of O_2. Basal liquid medium consisted of F-medium, described previously (23), which contained the following constituents in grams per liter of demineralized water: Na_2CO_3, 3.0; Na_2HPO_4, 0.6; NH_4Cl, 0.5; cysteine-HCl · H_2O, 0.25; Na_2S · $9H_2O$, 0.25; $MgCl_2$ · $6H_2O$, 0.1; $CaCl_2$ · $6H_2O$, 0.1; and resazurin, 0.001. In addition, 1% (vol/vol) each of vitamin and trace element solutions was added (28). The disaggregating medium consisted of basal medium with the following additions (grams per liter): $MgCl_2$ · $6H_2O$, 10.07; KCl, 0.76; and $CaCl_2$ · $2H_2O$, 0.04. Methanol, trimethylamine hydrochloride, or sodium acetate was added as a substrate at a final concentration of 0.05 M, and methanol was the growth substrate unless otherwise indicated. The final pH of the media was 6.8. The final osmolarity of disaggregating medium was varied by adjusting the NaCl concentration from 0.05 (basal concentration) to 1.0 M as desired. Media were dispensed into culture tubes (18 by 150 mm) or serum bottles (54 by 107 mm) and sealed with butyl rubber septa secured by aluminum crimp collars (Bellco Glass, Inc., Vineland, N.J.).

Aggregated and single cell forms of *Methanosarcina* spp. were maintained at 20°C in liquid basal medium and 0.4 M NaCl disaggregating medium, respectively, and transferred to fresh medium (10%, vol/vol) every 2 months. For long-term storage, cells were concentrated 10-fold and resuspended in basal medium or 0.4 M NaCl disaggregating medium that contained 50% glycerol (vol/vol) in 5-ml serum vials. The cell suspensions were stored in a freezer at −70°C. Frozen stocks were recovered by inoculating thawed aliquots into fresh medium (10%, vol/vol).

Colony plating procedures. Solidified medium consisted of liquid disaggregating medium without $NaHCO_3$, Na_2HPO_4, Na_2S · $9H_2O$, and cysteine-HCl · H_2O. NaCl was added to a final concentration of 0.4 M, unless otherwise indicated. Purified agar (1.25 or 0.5% [wt/vol]) was added for bottom agar or overlay agar, respectively. The medium was brought to a boil and cooled to 50 to 55°C under a flow of N_2-CO_2 (4:1). $NaHCO_3$ (0.38%, wt/vol), cysteine-HCl · H_2O (0.025%, wt/vol), and either trimethylamine, methanol, or sodium acetate were added before adjusting the pH of the medium to 6.8 with concentrated HCl. The medium vessel was then sealed with N_2-CO_2 in the headspace by using a butyl rubber stopper and sterilized by autoclaving.

The remaining steps were conducted in an anaerobic glove box (Coy Manufacturing Co., Ann Arbor, Mich.) that contained an atmosphere of N_2-CO_2-H_2 (15:4:1). Low oxygen tension (<6 ppm) was maintained in the anaerobic chamber with a palladium catalyst and H_2 (5%, vol/vol) in the chamber atmosphere. Hydrogen and oxygen partial pressures were constantly monitored with a gas analyzer (Coy Laboratory Products, Ann Arbor, Mich.). The gas mixture was periodically monitored with a gas chromatograph equipped with a stainless steel column (0.32 by 328 cm) that contained 100/120-mesh Carbosieve S-II (Supelco, Inc., Bellefonte, Pa.) and a thermal conductivity detector. All plasticware and glassware were equilibrated in the anaerobic chamber for at least 24 h before use.

Sterile molten medium was transferred to the anaerobic glove box and cooled to 50 to 55°C in a water bath incubator filled with glass beads instead of water. A sterile solution of Na_2HPO_4 (60%, wt/vol) was added to the medium (final concentration, 0.187% [vol/vol]) prior to dispensing. Bottom agar (30 ml) was poured into sterile petri plates (100 by 15 mm) and allowed to solidify at room temperature. Moisture condensation, which often caused confluence of the colonies, was minimized by predrying the solidified agar medium in the anaerobic chamber overnight prior to inoculation.

For plating, cultures that contained approximately 10^8 cells per ml in mid-exponential growth were transferred to the anaerobic chamber and serially diluted in liquid disaggregating medium. Diluted cells (0.1 ml) were added to 3 ml of molten overlay medium (50°C), mixed, and then layered over the solidified bottom agar. Plates were dried for approximately 30 min in the anaerobic chamber before being inverted and placed into a stainless steel anaerobic culture container (Torbal model AJ-3, Torsion Balance Co., Clifton, N.J.). A petri dish that contained anhydrous pellets of $CaCl_2$ was included in the anaerobic container to minimize condensation. An H_2S generator, consisting of a serum vial (23 by 47 mm) that contained a premeasured amount of Na_2S · $9H_2O$, was attached to the anaerobic container with a sidearm perforated through a septum. The anaerobic container was sealed and removed from the anaerobic chamber. The H_2S generator was activated by injecting concentrated H_2SO_4 through the septum into the vial. The anaerobic container was then incubated at the desired temperature. To inspect the plates, H_2S was purged from the anaerobic container with N_2-CO_2 in a fume hood. The container was then transferred into the anaerobic chamber before opening. Plates could be reincubated by replenishing the H_2S generator with Na_2S · $9H_2O$ and repeating the steps described above.

Viable cells were also enumerated in roll tubes by inoculating serial dilutions of cultures into anaerobe tubes (25 by 150 mm; Bellco Glass, Inc.) that contained 10 ml of molten bottom medium (50°C) and the appropriate growth substrate. The tubes were sealed under N_2-CO_2 (4:1) with butyl rubber stoppers and rolled with a tube spinner (Bellco Glass, Inc.) until the agar solidified (2).

Analytical methods. Growth of disaggregated single cells in liquid cultures was monitored by measuring the increase in A_{550} (path length, 18 mm) with a Spectronic 21 spectrophotometer (Bausch and Lomb). Direct cell counts were made

with a Petroff-Hausser counting chamber (C. A. Hausser and Son, Philadelphia, Pa.) as described previously (14). In order to enumerate cells in multicellular aggregates, the heteropolysaccharide matrix was enzymatically removed by a modification of the procedure described previously (16) which employs an endopolysaccharide hydrolase excreted by *M. mazei* LYC. A mid-exponential-phase culture of the aggregated species to be enumerated was harvested by centrifugation, and the cell pellet was resuspended in filter-sterilized culture supernatant from *M. mazei* LYC grown to stationary phase. Spermine was included in the supernatant at a final concentration of 1 mM to stabilize the disaggregated cells (23). All disaggregation steps were performed in an anaerobic chamber. Cells were completely disaggregated after incubation for 60 min at room temperature and counted directly.

Glucuronic acid was measured by a modification of the carbazole method (16) and reported as micrograms of uronic acids per 10^8 cells.

Isolation of chromosomal DNA. Disaggregated cells were equilibrated in 0.4 M NaCl disaggregating medium. Cultures were then harvested by centrifugation and resuspended in TE-salts buffer (0.01 M Tris-HCl–0.1 mM EDTA plus 0.05 M $MgSO_4$ and NaCl equimolar to NaCl in growth medium) to a final concentration of 10^{10} cells per ml. TE-salts buffer stabilizes the S-layer and prevents osmotic lysis of cells. Cells were then lysed by the addition of sodium dodecyl sulfate (SDS) (10%, wt/vol) to a final concentration of 0.01%. Slow addition of the SDS while the cell suspension was mixed prevented clumping during cell lysis. DNA was extracted with equal volumes of phenol (equilibrated with Tris, pH 8.0) until a precipitate was no longer observed at the aqueous-phenol interface (20). Sodium acetate was added to a final concentration of 0.3 M. The DNA was precipitated from the lysate with 5 volumes of ethanol and spooled on a glass rod as described by Marmur (20). The DNA was washed in 70% ethanol and redissolved in TE buffer without $MgSO_4$ and NaCl.

Microscopy. Light micrographs were made with an Olympus BH-2 phase-contrast microscope. For thin-section electron micrographs, cells were fixed with 2% glutaraldehyde and 2% osmium tetroxide and dehydrated in a graded series of ethanol mixtures. Cells were embedded and sectioned in resin and then poststained with uranyl acetate and lead citrate (22). Thin-section micrographs were made with a Hitachi H7000 transmission electron microscope.

Chemicals. Purified agar was obtained from Difco Laboratories, Detroit, Mich. All other chemicals were of reagent grade.

RESULTS

Transition of *Methanosarcina* spp. from aggregates to single cells. *M. barkeri* MS, 227, UBS, Fusaro, and W, *M. vacuolata* Z-761, *M. mazei* S-6 and LYC, and *M. thermophila* TM-1 grown as aggregated cells in basal medium were transferred to disaggregating medium containing 0.05 M NaCl. Strains were then adapted for growth in higher extracellular solute concentrations by sequentially transferring late-exponential-phase cultures (10%, vol/vol) into disaggregating media that contained increasing NaCl concentrations (i.e., 0.1, 0.2, 0.4, 0.8, and 1.0 M). Concomitant with adaptation and growth in media with NaCl concentrations above 0.4 M, all aggregated strains of *Methanosarcina* spp. underwent a transition to single cells (Fig. 1). *M. thermophila* TM-1, previously reported to disaggregate in marine medium, formed single cells in 0.4 M NaCl. While some of the strains (i.e., *M. barkeri* MS and UBS or *M. mazei* S-6 and LYC) disaggregated at NaCl concentrations as low as 0.4 M, the remaining strains did not disaggregate below 0.8 M. This transition to single cells was coincident with an increase in culture turbidity. Disaggregation to single cells was also observed when equivalent osmolar concentrations of LiCl, Na_2SO_4, or sucrose were substituted for NaCl. However, a high concentration of Mg^{2+} (>0.2 M) in disaggregating medium did not promote transition to single cells. This process was independent of the substrate used; single cells could be maintained in disaggregating medium that contained trimethylamine, methanol, or acetate.

Disaggregated single cells could be readapted to grow in 0.05 M NaCl by sequentially transferring cultures into media with decreasing NaCl concentrations, provided that 0.05 M $MgCl_2$ was included. Although single cells remained viable and grew at NaCl concentrations below 0.2 M, they were larger and more spherical than the irregularly shaped cells observed in NaCl concentrations above 0.2 M. Disaggregated single cells grown with NaCl below 0.2 M were also more prone to autolysis as cultures approached the stationary phase.

Single cells grown in disaggregating medium with 0.05 M NaCl could be transformed back to aggregated cells by sequentially transferring cultures into media with decreasing amounts of $MgCl_2$ (i.e., 0.05, 0.025, 0.01, and 0.005 M). Cell aggregates were formed after 2 to 4 weeks in medium containing <0.005 M Mg^{2+}. *M. acetivorans*, which was isolated from a marine source as disaggregated single cells, also formed multicellular aggregates when adapted to medium containing 0.05 M NaCl and <0.005 M Mg^{2+} (Fig. 1e and f).

Cell ultrastructure. Thin-section electron micrographs of *Methanosarcina* spp. showed that aggregated cells grown in basal medium were surrounded by an amorphous outer layer and an inner monolayer adjacent to the cell membrane (Fig. 2a and d). The relatively thick (<100 nm) outer layer is composed primarily of glucuronic acid and galactosamine heteropolysaccharide (i.e., methanochondroitin [15]), and the thin (10 nm) inner monolayer or S-layer is composed primarily of protein (1, 25). Following transition of aggregated cells to disaggregated single cells, *M. barkeri* MS retained the protein monolayer but not the methanochondroitin outer layer (Fig. 2b and e). Addition of SDS (0.01% [wt/vol] final concentration) lysed single cells, which is consistent with the loss of the methanochondroitin outer layer (6, 19, 23). Immediately prior to disaggregation, there was a reduction in the thickness of the methanochondroitin outer layer (Fig. 2c and f).

Glucuronic acid, which comprises 30% of the methanochondroitin synthesized by *M. barkeri* (15), was measured in cell pellets and supernatants of aggregated and disaggregated cultures of *M. barkeri* MS. Uronic acid levels of aggregated cultures were 12.3 ± 2.3 and 1.3 ± 0.3 μg per 10^8 cells in cell and supernatant fractions, respectively; and cell and supernatant fractions of disaggregated single cell cultures were 1.25 ± 0.5 and 1.1 ± 0.6 μg per 10^8 cells, respectively.

Growth properties of *Methanosarcina* spp. as aggregated and as individual cells. The aggregated and single-cell forms of *Methanosarcina* spp. exhibited different growth properties. A high concentration of Mg^{2+} (>0.05 M) was not required for growth of aggregated cells, nor did the presence of up to 0.4 M $MgCl_2$ adversely affect their growth. In contrast, the disaggregated single cell form of *Methanosarcina* spp. required at least 0.05 M Mg^{2+} for maximum growth

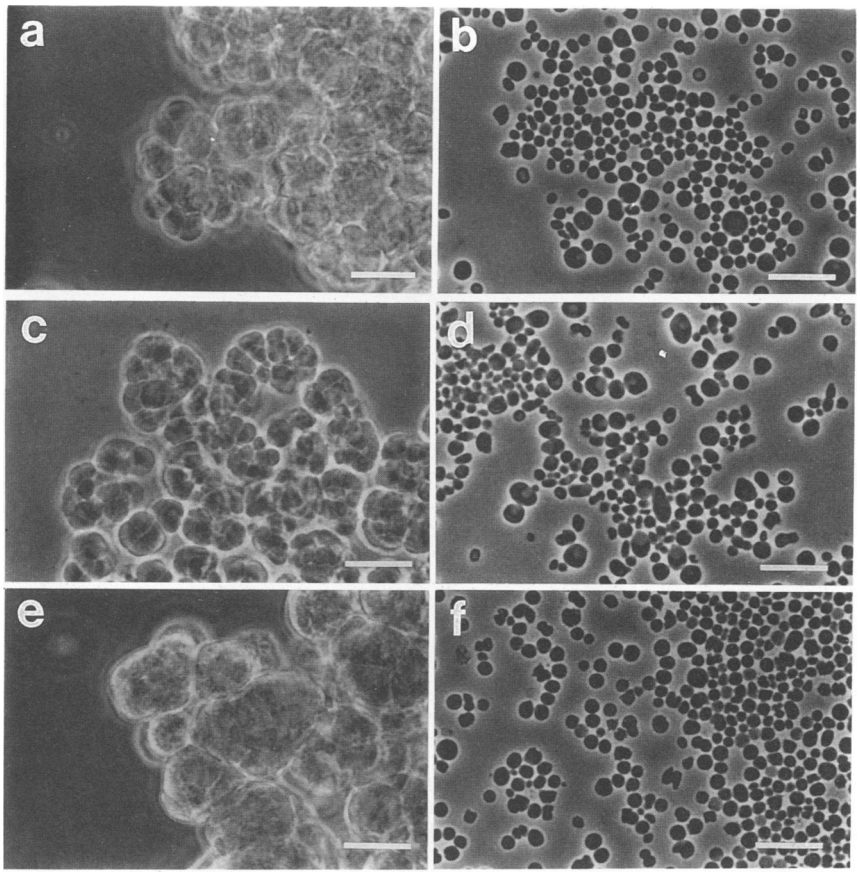

FIG. 1. Phase-contrast micrographs of *M. barkeri* MS (a and b), *M. mazei* S-6 (c and d), and *M. acetivorans* C2E (e and f) grown in basal medium and disaggregating medium that contained 0.05 and 0.4 M NaCl, respectively. All species tested grew as multicellular aggregates in basal medium containing 0.05 M NaCl and disaggregated into individual cells in disaggregating medium containing 0.4 to 1.0 M NaCl. Bars, 10 μm.

(Fig. 3), and suspension of cells from disaggregating medium directly into medium containing <0.005 M Mg^{2+} caused cell lysis. The high Mg^{2+} requirement by disaggregated single cells could be circumvented by substituting the polyvalent amine spermine (1 mM).

The effect of temperature on the growth of aggregated cells and disaggregated single cells was examined (Fig. 4). Cultures of aggregated and disaggregated cells were each adapted to grow in disaggregating medium containing 0.2 M NaCl. The highest growth temperature of the aggregated forms of *M. barkeri* (Fig. 4), *M. mazei*, and *M. acetivorans* strains (data not shown) was 45°C, whereas the maximum growth temperature for the single cells of each strain was 40°C. The growth rates of the aggregated forms were approximately one-half to two-thirds those of the single-cell forms at each temperature tested.

Disaggregated single cells of all 11 *Methanosarcina* strains were stable during culture storage; they could be maintained in liquid disaggregating medium containing 0.4 to 1.0 M NaCl for at least 2 months without transfer. Frozen cells stored at −70°C in medium containing 50% glycerol remained viable after storage for 5 years. When frozen stock cultures were thawed and inoculated into fresh disaggregating medium, there was a 1-to-3-week lag period before growth was observed.

Growth of single cells as colonies on solid medium. Conditions were optimized for growth of disaggregated *M. barkeri* MS, *M. thermophila* TM-1, *M. mazei* S-6, and *M. acetivorans* C2A on solidified medium. Several factors affected the plating efficiency of *Methanosarcina* spp., judged on the basis of a comparison of CFU on plates, CFU in roll tubes, and direct cell counts. Since the CFU in roll tubes were equal to the direct cell counts, growth efficiencies on plates were based on the percent CFU of roll tube cultures.

Plating inocula were prepared by growing cultures to mid-exponential growth (10^7 cells ml^{-1}) in liquid 0.4 M NaCl disaggregating medium. Pouring inocula in a 0.5% agar overlay resulted in maximum plating efficiencies (>95%), while spreading cultures on the surface of bottom agar yielded lower plating efficiencies (<10%). Colonies (~2 mm in diameter) of all four species were observed within 5 days when they were grown on medium that contained either trimethylamine or methanol and within 14 days on medium that contained acetate. Colonies of *Methanosarcina* spp. grown with any of these substrates could be spotted, streaked, or replica plated by using a velvet-covered block.

Media containing NaCl concentrations ranging from 0.4 to 0.6 M yielded >95% plating efficiency, while lower plating efficiencies of 45 and 72% occurred with 0.2 and 0.8 M NaCl, respectively. An H_2S concentration between 0.5 and 0.8% (vol/vol) in the gas phase of the anaerobic container yielded >95% plating efficiency, while no growth was observed at

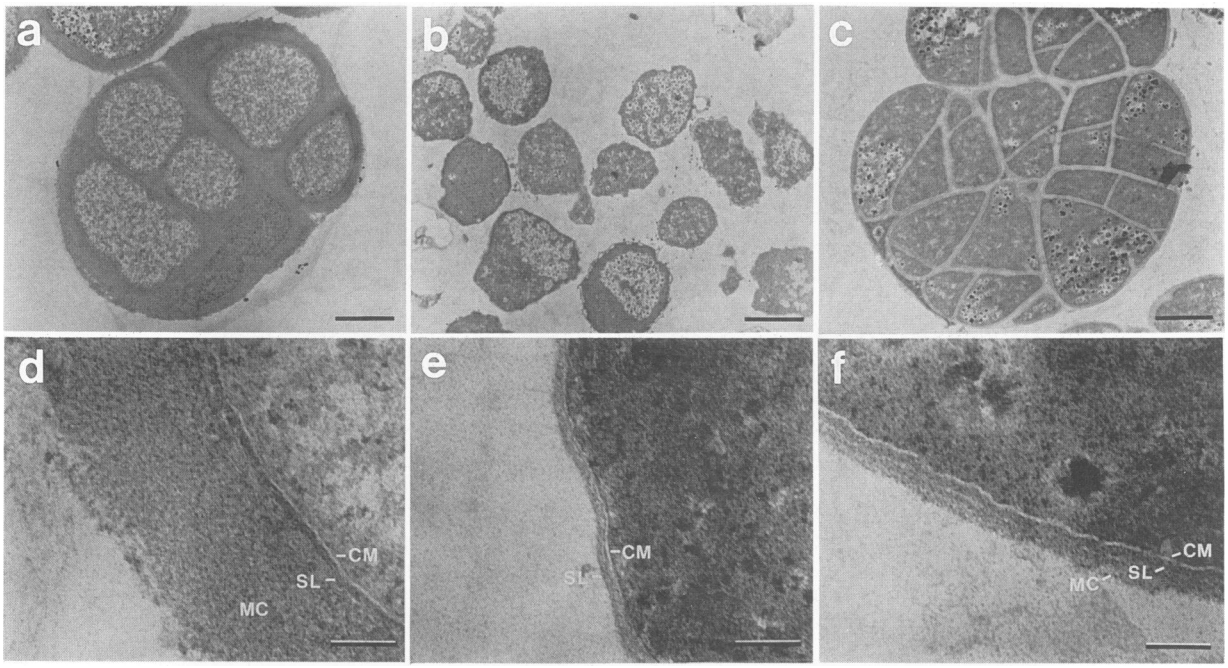

FIG. 2. Thin-section electron micrographs of *M. barkeri* grown in 0.05 and 0.4 M NaCl. The micrograph of *M. barkeri* MS grown in basal medium (a) shows the close association of cells in a methanochondroitin matrix and *M. barkeri* MS grown in disaggregating medium containing 0.4 M NaCl (b) shows individual cells that lack the methanochondroitin outer cell layer, while *M. barkeri* 227 grown in 0.4 M NaCl (c) retained a thinner methanochondroitin matrix than cells grown without NaCl. *M. barkeri* 227 completely disaggregated in disaggregating medium containing 0.8 M NaCl (not shown). Bars, 1 μm. High-magnification thin-section electron micrographs of cell-medium interfaces of *M. barkeri* MS grown in 0.05 M (d) or 0.4 M (e) NaCl and *M. barkeri* 227 grown in 0.4 M NaCl (f) show the cytoplasmic membrane (CM), protein S-layer (SL), and the methanochondroitin outer layer (MC). Bars, 0.1 μm.

above 1.1%. Incubation temperatures of 30 to 40°C resulted in >95% plating efficiency, while significantly lower growth efficiencies (<50%) were observed outside of this temperature range. A minimum bottom agar volume of 30 ml resulted in >95% plating efficiency and maximum colony size, while lower volumes resulted in efficiencies below 50%.

Isolation of DNA. High-molecular-weight chromosomal DNA was readily prepared from all *Methanosarcina* spp. after the growth of cultures as detergent-sensitive single cells in disaggregating medium. Cells were gently lysed by the addition of SDS (final concentration of 0.01%, wt/vol) to a

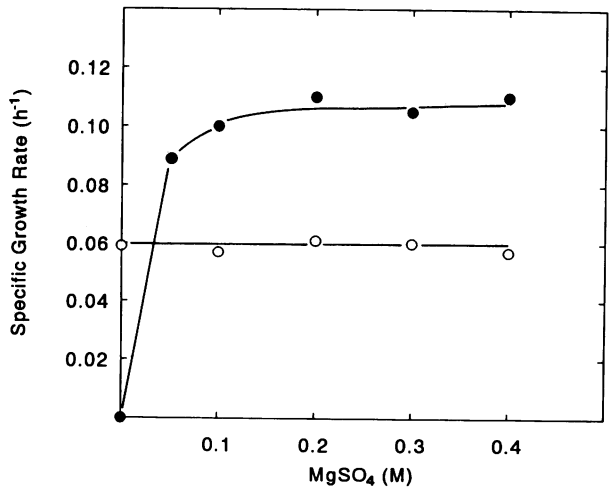

FIG. 3. Effect of magnesium ion concentration on the growth rate of *M. barkeri* MS grown as aggregated (○) and disaggregated cells (●). Aggregated and disaggregated cells were grown in disaggregating medium that contained 0.2 M NaCl. Growth was monitored by measuring methane production.

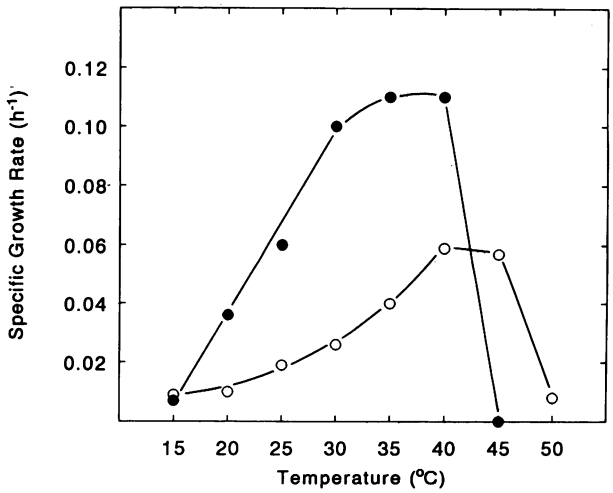

FIG. 4. Effect of temperature on the growth rate of *M. barkeri* MS grown as aggregated (○) and disaggregated (●) cells. Aggregated and disaggregated cells were grown in disaggregating medium that contained 0.2 M NaCl. Growth was monitored by measuring methane production.

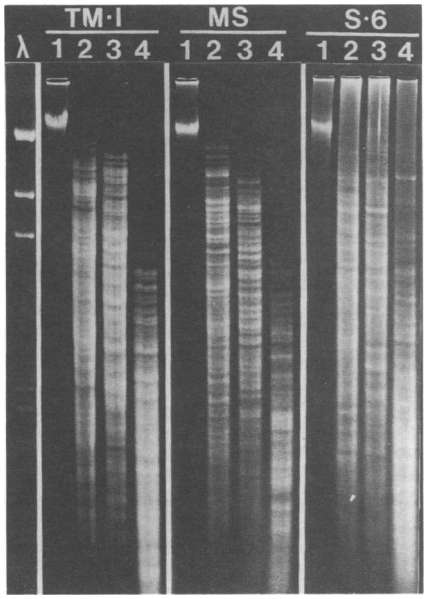

FIG. 5. Agarose gel electrophoresis of whole and restriction enzyme-digested chromosomal DNA from *M. thermophila* TM-1, *M. barkeri* MS, and *M. mazei* S-6. Cultures were grown in marine medium as individual cells, harvested by centrifugation, lysed with SDS, and purified as described in Materials and Methods. Lane λ, *Hin*dIII-digested lambda DNA included as a size standard. Lanes 1, undigested chromosomal DNA (0.2 μg) from each strain showing the lack of sheared DNA in the preparations. Lanes 2 through 4, complete digests of chromosomal DNA (10 μg) with *Acc*I, *Eco*RI, and *Hae*III, respectively.

concentrated cell suspension. Chromosomal DNA was then isolated by standard methods (see Materials and Methods). This procedure reproducibly yielded intact high-molecular-weight material with minimal shearing (Fig. 5).

DISCUSSION

M. thermophila TM-1 has previously been shown to adapt and grow in medium containing NaCl concentrations ranging from 0.05 to 1.0 M (23). In this study, 10 additional strains representing five described *Methanosarcina* spp., including the marine species *M. acetivorans*, were adapted to grow over a similar range of NaCl concentrations. Concomitant with the adaptation to high osmolarity (>0.4 M NaCl), all strains grew as single cells rather than as multicellular aggregates. Previous reports showed that *M. mazei* could be disaggregated by high concentrations of substrate or divalent cations (6, 16, 30). However, the disaggregation process in the present study occurred independently of the concentration or type of energy substrate and was not mediated by divalent cations. These observations indicate that the transition phenomenon reported here results from high extracellular solute concentration. This contradicts our earlier report that disaggregation of *M. thermophila* was cation dependent because growth was inhibited in sucrose (23). The earlier conclusion likely resulted because an equimolar amount of sucrose was substituted for NaCl, causing the cells to undergo osmotic shock in the more hypotonic sucrose medium. In this study, the ability to promote disaggregation with a high concentration of either NaCl or sucrose indicates that the disaggregation process results from osmotic pressure created by extracellular solute rather than an ion-specific stimulus.

Growth of *Methanosarcina* spp. as disaggregated single cells rather than aggregated cells results from the absence of a methanochondroitin outer layer, as evidenced in electron micrographs (Fig. 2). Methanochondroitin is composed of glucuronic and galacturonic acids and *N*-acetyl-D-galactosamine, which comprise approximately 7 to 20% and 20 to 40%, respectively, of the heteropolysaccharide by dry weight (15). The 20-fold reduction of uronic acid synthesis in cultures of disaggregated single cells, when compared with cultures of aggregated cells, suggests that disaggregation results from a cessation in synthesis of one or more monomers of the methanochondroitin. This conclusion is supported by the observation that, prior to disaggregation, there is a gradual reduction in the thickness of the methanochondroitin layer as cells are adapted to higher NaCl concentrations (Fig. 2c and f). An alternative explanation, that the single cells are mutants with defects in methanochondroitin synthesis or assembly, is unlikely because the single cell form should not have returned to the aggregated form when adapted to medium containing low NaCl and Mg^{2+} concentrations.

Cells that lack the methanochondroitin outer layer grow as irregular cocci in medium containing NaCl concentrations of 0.2 M and greater (Fig. 1). Although single cells can be adapted to grow and remain viable in medium that contains NaCl in concentrations below 0.2 M, the cells are larger and more spherical and more prone to lyse than cells grown in medium containing higher NaCl concentrations. Harris (6) also reported enhanced stability of *M. mazei* single cells in medium made hypertonic with 0.3 M sucrose. The conclusion that *Methanosarcina* spp. become hypertonic relative to the extracellular solute concentration in medium containing less than 0.2 M NaCl is supported by previous studies which reported that the aggregated form of *Methanosarcina* spp. grown in low osmolar medium (<0.05 M NaCl) contains a higher concentration of intracellular osmolytes relative to the medium (8, 18, 24a). These observations suggest that the additional physical stability provided by the methanochondroitin outer layer may function to protect cells from excessive turgor pressure caused by hypotonic environments such as freshwater lakes and rivers.

The methanochondroitin outer layer also appears to promote cell stability during growth in low (0.005 M) concentrations of divalent ions such as Mg^{2+} (Fig. 3). Previous reports indicate that polyvalent cations such as Mg^{2+} or spermine are required to maintain the integrity of protein subunits that compose the S-layer (15, 23, 25, 27). In contrast, aggregated cells that synthesize a methanochondroitin outer layer do not require high extracellular concentrations of divalent cations to maintain the integrity of the cells (Fig. 3). Kreisl and Kandler (15) suggested that the rigid heteropolysaccharide outer layer may have a polymer-binding function analogous to that of chondroitin, in this case binding with the charged S-layer of the *Methanosarcina* spp. The net positive charge created by the galactosamine component of the heteropolysaccharide outer layer may have an electrostatic effect on the protein subunits similar to the effect of polyvalent cations, thereby negating or reducing the requirement for Mg^{2+} to stabilize the S-layer. The results of this study support this hypothesis.

The growth properties of *Methanosarcina* spp. (aggregated versus disaggregated single cells) differ with respect to growth rate and maximum temperature tolerance. Single-cell cultures of *Methanosarcina* spp. grow at faster rates than

cultures of aggregated cells. The greater growth rates of single cells appear to be due to the absence of a methanochondroitin outer layer rather than a physiological preference for high concentrations of NaCl, since different growth rates for the two forms were observed at each NaCl concentration tested (Fig. 3 and 4). The higher growth rate observed for the single cells may be attributed to the energy conserved by not synthesizing heteropolysaccharide, which constitutes approximately 10% of the cell dry weight of aggregated cells (12). Alternatively, the faster growth of single cells may be due to the greater surface-to-volume ratio than for aggregated cells, which would affect the rate of nutrient uptake. Another difference observed between the two forms was that single cells had a maximum temperature tolerance 5°C below that of the aggregated form. The higher temperature tolerance of the aggregated cells observed in this study and previously with *M. thermophila* (23) suggests that the heteropolysaccharide outer layer provides additional temperature tolerance to *Methanosarcina* spp.

Harris (6) reported high plating efficiencies for *M. mazei* on solidified medium that contained 0.3 M sucrose as an extracellular solute. In this study, *M. mazei* and three additional *Methanosarcina* spp. were plated as single cells on agar-solidified medium that contained NaCl as an extracellular solute. Factors critical for the efficient growth of *Methanosarcina* spp. on agar-solidified medium were identified to optimize plating efficiency at >95%. The effective range of H_2S was 0.5 to 0.8% (vol/vol), which is similar to the optimum range reported for other genera of methanogens (11, 29). The effect of NaCl concentrations on the colonization efficiency of *Methanosarcina* spp. on agar-solidified medium paralleled the effect of NaCl on the growth rates of *M. thermophila* in liquid cultures (23).

The transition from aggregated to single cells also facilitated isolation of intact high-molecular-weight chromosomal DNA with minimum shearing (Fig. 5) (6). Aggregated cells of *Methanosarcina* spp. require harsh mechanical methods for lysis that tend to yield sheared chromosomal and plasmid DNA. The single-cell form of *Methanosarcina* spp. grown in disaggregating medium can be gently lysed with detergent, and intact chromosomal DNA is then isolated by standard methods (6). In addition, this technique has been effectively used for screening plasmid DNA in *Methanosarcina* spp. (6, 24).

In this study, we have demonstrated global osmotic disaggregation of *Methanosarcina* spp. and described conditions for the growth of these species as viable, disaggregated cells. Apparent anomalies among *Methanosarcina* spp. have been the diverse morphologies exhibited by irreversibly aggregated or disaggregated single-cell species and the inclusion of obligately marine and nonmarine species within a closely related phylogenetic group (25). However, the results of this study show that when *Methanosarcina* spp. from nonmarine sources disaggregate as the extracellular solute concentration is increased, their morphology becomes similar to that of the marine species *M. acetivorans* and *M. frisia* (3, 21). The ability of all species from both marine and nonmarine sources to express a reversible single-cell phenotype and tolerate a similar range of extracellular solute concentrations is consistent with the close phylogenetic relationship among *Methanosarcina* spp. The expression of these characteristics by *Methanosarcina* spp. suggests that this group of methanogens has evolved physiological and structural adaptations that enable them to be halotolerant and proliferate in an osmotically diverse range of environments.

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